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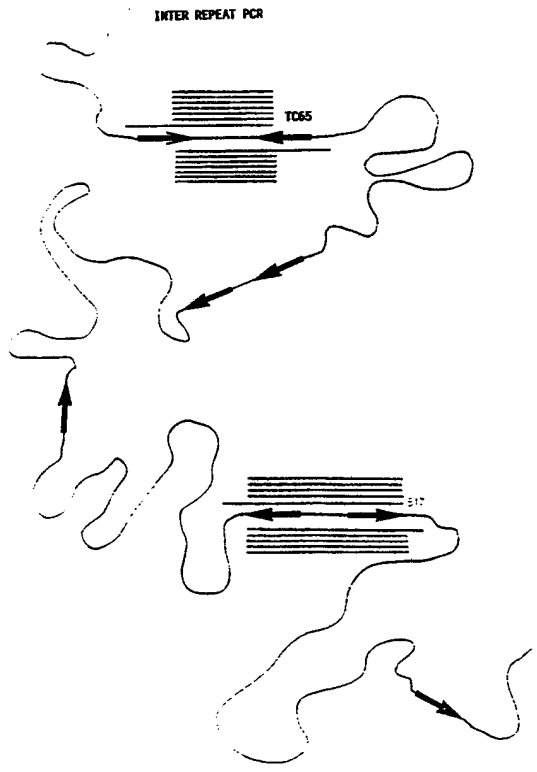
(54) Title: METHOD OF DETECTING DNA SEQUENCE VARIATION

(57) Abstract

Detection of genetic variation by 2-D electrophoresis of DNA fragments of a DNA sample to be analysed, the fragments being separated in one dimension according to length and in the other dimension according to base sequence, transfer to a membrane filter and hybridization analysis using DNA or RNA probes. The DNA fragments consist of inter-repeat sequences generated on the basis of the DNA to be analysed by means of a DNA amplification process such as a PCR with repeat-specific primer(s). Suitable inter-repeat sequences are for example sequences located between two Alu-repeats, between two Kpn-repeats, or between an Alu- and Kpn-repeat. Preferably inter-repeat sequences generated by means of a DNA amplification process with repeat-specific primer(s), performed on subgenomic DNA, for example DNA of one chromosome or a part thereof, are used as a probe in the hybridization analysis.



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TITLE: Method of detecting DNA sequence variation.

This invention relates to a method of detecting genetic variation by subjecting DNA fragments, derived from a DNA sample to be analyzed, to a two-dimensional electrophoretic separation, with the fragments being separated in one dimension on the basis of differences in length and in the other dimension on the basis of differences in base sequence, transferring the resultant separation pattern to a membrane filter and subjecting the transferred separation pattern to a hybridization analysis using one or more DNA or RNA probes.

Such a method is used in the field of so-called DNA diagnostics, i.e. the use of methods for demonstrating genetic variation at those sites which may be an indication for certain properties, such as susceptibility to certain diseases and other medically and economically important characteristics. The term "genetic variation" refers to differences between individuals in the base pair sequence of their DNA.

Genetic variation can be detected in different ways. One well known method involves hybridization analysis after electrophoretic separation of DNA fragments, obtained after treating genomic DNA with a restriction enzyme (Southern blot hybridization analysis; Southern, 1975). Hybridization with a specific labelled DNA fragment (probe) results in one or more select bands. Each band represents a DNA fragment that hybridizes with the probe used. The position of the bands is a measure for the size of the DNA fragments which they represent. When for a given probe the position of the bands generated therewith varies between individuals as a result of deletions, insertions and/or the presence or absence of a recognition site for the restriction enzyme used, this is called restriction fragment length polymorphism (RFLP). Experience has taught that some regions in the genome (the total of hereditary information) of humans, animals or plants are much more polymorphic than other regions. Probes for such regions may be extremely

useful as markers for certain hereditary properties. Thus, for example, in a human family in which a certain hereditary disease occurs, the inheritance of the disease, together with a variant of a polymorphic DNA sequence, is an indication that the defective gene involved in the disease is located in close proximity thereto; this is called genetic linkage. The variant of the polymorphic DNA sequence in question may then serve as a diagnostic marker for the disease in question. Further, the disease gene can be isolated using the marker.

Probes which detect polymorphic DNA sequences can be used in a comparable manner in plant improvement and in the breeding of cattle and other economically important animals. The point is that if it is known that a particular DNA variant is always inherited together with a particular property, the presence or absence of that property can be determined at an early stage which is of great importance for improvement because in this way the generation interval can be avoided (this principle is called "Marker Assisted Selection").

In order to link as many hereditary properties as possible to polymorphic markers, it is naturally of importance to have some information about the location of those markers, regarding both their relative position and their exact position on the chromosomes. If for humans and a number of important plant and animal species genetic marker maps were available, with a marker for each approx. 2 million base pairs, linking markers to hereditary properties would be a routine affair. However, such maps are not as yet available; such maps as are available, are incomplete and/or consist largely of markers which are not always equally informative, i.e. are not very polymorphic (see for instance Donis-Keller et al., 1987). Even with such an accurate map, moreover, it will still involve a lot of work to link a particular hereditary characteristic to a particular marker because each marker must be examined separately for linkage with a particular characteristic. Since the human genome comprises approx. 3×10^9 base pairs, this means for this species, for instance, that some 1500 markers would have to be tested each time a hereditary property is to be mapped.

Recently, we have proposed a method which enables a great number of hyperpolymorphic DNA sites to be analyzed simultaneously by means of an electrophoretic separation in two dimensions, followed by transfer of the separation pattern to membrane filters and hybridization with so-called minisatellite core probes (EP-A-0 349 024; Uitterlinden et al., 1989; Uitterlinden and Vijg, 1989). In this electrophoretic separation of DNA fragments in two dimensions, use was made of a previously developed system in which separation by size is followed by separation on the basis of base pair composition, at right angles to the direction of the first separation (Fischer and Lerman, 1979). Separation on the basis of base pair composition was realized by Fischer and Lerman by using so-called denaturing gradients (Fischer and Lerman, 1979; Fischer and Lerman, 1983). In that method, the double-stranded DNA migrates in an electric field in a gel (for example, a polyacrylamide gel) in which beforehand a gradient of denaturant (a mixture of urea and formamide) has been provided. During their migration the DNA fragments meet with an increasingly higher concentration of the denaturant and will eventually melt at a certain point in the gradient. This point is highly dependent on the base pair composition of the DNA fragment (see Lerman et al., 1984). We have demonstrated that separation of DNA fragments (obtained *inter alia* by restriction enzyme digestion of human DNA) in two dimensions on the basis of the above principle, followed by transferring the separation pattern to membrane filters and hybridization with GC-rich minisatellite core probes, leads to a pattern in which, depending on the probe used, 300-700 alleles of loci can be distinguished as spots (see Fig. 1). Our data show that approx. 20-50% of these loci belong to the hyperpolymorphic type which is so characteristic for VNTR-type (variable number of tandem repeats) loci (Nakamura et al., 1987). This means that in this manner approx. 60-350 alleles and hence 30-175 hyperpolymorphic loci can be screened simultaneously. Rehybridization with other GC-rich core probes results in comparable patterns in which most spots will correspond with loci in the genome other than

the loci which were detected with the first probe. In other words, the different core probes detect different groups of loci, although some overlap is demonstrable (5-10% on average). Using the above-described method (2-D DNA typing), therefore, within a short time a large number of hyperpolymorphic loci in a genome can be screened, which enables ready mapping of hereditary characteristics. As such the system is a step forward relative to Southern hybridization analysis, which can almost be called classic.

It seemed to us that 2-D DNA typing as an efficient measuring system in genetic studies could be further refined considerably and could be applied more specifically if the following drawbacks could be overcome:

(1) The sequences detected by the core probes are irregularly distributed within the genome. For example, minisatellite VNTR-type sequences occur in humans much more often at the telomeres (terminal ends of the chromosome) than elsewhere (Royle et al., 1989). As to other VNTR-type sequences, not much is known about this and also in animal species other than man and in plants there is only little information about the occurrence and distribution of VNTR-type sequences.

(2) The 2-D DNA typing method is non-specific; the region in the genome that is covered with certain core probes is not known a priori. It is therefore necessary once a spot has been identified to determine the genomic location of each spot. Although time-consuming, this is certainly feasible and considering the frequent occurrence of VNTR-type sequences it may be assumed that with a proper combination of core probes and a good strategy for locating the spots, eventually a fine-meshed genomic map can be constructed (see also EP-A-0349024). The time involved, however, will remain considerable.

(3) A third point concerns the fact that only 20-50% of the sequences detected by the core probes are polymorphic and, accordingly, useful for the analysis. In other words, the majority of the spots cannot be used for mapping and only takes up space in the separation pattern.

It would therefore be much better to use sequences which occur once at least about every 2 million base pairs, are nearly always polymorphic and are equally distributed over the genome. A sequence with these properties is for example the repetitive element Alu. However, we have previously demonstrated that hybridization of a two-dimensional separation pattern of mammalian DNA with Alu as probe results in an unstructured smear without any clear stains. This was explained, on the one hand by the great number of Alu copies per genome (approx. 700,000; for a consideration of the distribution of Alu and other repeats over the genome, see Schmid and Jelinek, 1982; Korenberg and Rykowski, 1988; Moyzis et al., 1989), and, on the other, by the not particularly GC-rich character of Alu. This last is in contrast with the sequences detected with the GC-rich core probes, which on the basis of their GC-rich character function as "clamp" (i.e. highest melting domain) during the separation in the denaturing gradient (2nd dimension separation) which results in a focussing as spot of this kind of sequences in the second dimension. Since the sequences located adjacent the VNTR will vary from locus to locus and moreover function as lowest melting domain, the resultant spots will permit good separation. However, many of the DNA fragments which contain Alu will melt more or less simultaneously and thus give rise to clustering in the gel (for an extensive consideration of this, see EP-A-0 349 024 and Uitterlinden and Vijg, 1989). What this comes down to, therefore, is that the number of Alu fragments per genome is too large for use as markers and their melt-out characteristics are not very suitable for the gel separation system used in 2-D DNA typing. This forms an obstacle to the use in hybridization experiments of Alu- or comparable repeats as "anchoring points" for a two-dimensional screening of the genome.

The fact that Alu occurs so frequently in the genome (every 10,000 base pairs on average), however, makes it possible to distinguish so-called inter-Alu regions and to isolate them directly using the polymerase chain reaction (PCR). Recently Nelson et al. (1989) have demonstrated that

inter-Alu regions can be amplified by means of PCR by using primers which are specific for Alu. For instance, by using the oligo TC65 as the single primer in the amplification reaction, in principle all regions of the human genome which are located between 2 Alu copies in reversed orientation, can be amplified. Fig. 2 is a diagrammatic overview of this method and Fig. 3 gives an overview of the binding sites on the Alu repeat of the suitable Alu-specific primers listed in Table 1.

TABLE 1: origin and sequence of inter-Alu specific primers

Nelson et al., 1989:	
TC65 (559)	5'-AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT-3'
517	5'-CGACCTCGAGATCT(C/T) (A/G)GCTCACTGCAA-3'
P. de Jong, personal communication:	
PDJ33	5'-GCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCA-3'
PDJ34	5'-TGAGCCGAGATCGCGCCA(T/C)TGCACTCCAGCCTGGG3'
unpublished:	
PDJ33A	5'-GGATCCGCCTCCCAAAGTGCTGGGATTACAGG-3'
PDJ34A	5'-GAATTCTGAGCCGAGATCGCGCCACTGC-3'
Cotter et al., 1990:	
PRIM IV	5'-CAGAATTCGCGACAGAGCGAGACTCCGTCTC-3'

The present invention provides a method for detecting genetic variation by subjecting DNA fragments derived from a DNA sample to be examined by two-dimensional electrophoretic separation, with the fragments being separated in one dimension on the basis of differences in length and in the other dimension on the basis of differences in base sequence, transferring the resultant separation pattern to a membrane filter and subjecting the transferred separation pattern to a hybridization analysis using one or more DNA or RNA probes, which method is characterized by using DNA fragments which consist of one or more inter-repeat sequences which on the basis of the DNA to be examined have been generated by means of a DNA amplification process such as the polymerase chain reaction (PCR) with repeat-specific primer(s).

A very important preferred embodiment of the invention is characterized in that in the hybridization analysis, as a probe use is made of one or more inter-repeat sequences which have been generated by means of an amplification process such as a PCR with repeat-specific primer(s), performed on subgenomic DNA, for instance DNA of one chromosome or a part thereof.

Thus, the invention makes it possible to detect genetic variation on specific chromosomes or parts thereof, using a selective amplification of double-stranded DNA in specific sites, occurring at least every 2 million base pairs, an electrophoretic separation of the amplification products in two dimensions, transfer of the separation pattern to a membrane filter, followed by a hybridization with labelled DNA fragments which may come from (a) cloned DNA such as cosmid and YAC (yeast artificial chromosome) clones, or (b) chromosomes or parts thereof such as obtained after flow-sorting chromosomes or such as are present in a human-hamster somatic cell hybrid.

The DNA to be examined will mostly consist of the DNA of a complete genome of an animal or plant, and the most important applications will concern human DNA.

Instead of total human DNA, it is also possible to start from DNA coming from a specific human chromosome or a part thereof. Individual chromosomes can be obtained by means of flow sorting, but most often this does not result in a completely pure chromosome population. Another method, employed more often, is to start from so-called hybrid cell lines. In that method, for instance a human cell line is fused with a hamster cell line. In most cases, after the fusion of the nuclei, the human chromosomes will nearly all be removed. This makes it comparatively easy to obtain human-hamster hybrid cell lines with only one human chromosome or a part thereof. Now, by starting from DNA that comes from a human-hamster hybrid cell line, in principle all inter-Alu regions which are located on a specific human chromosome or a part thereof can be selectively amplified. This has been described extensively by Nelson et al. (1989). The importance of this procedure relates to the new possibilities it provides of obtaining DNA fragments from

certain defined parts of the genome. These DNA fragments can then be used as probes and/or be analyzed. This is explicitly mentioned by Nelson et al. (1989) (see also Ledbetter et al., 1990a and b; Lichter et al., 1990).

It seemed to us that inter-Alu PCR, coupled with 2-D DNA typing, could be used for genome scanning in a way that is superior in at least two respects to the previously described genetic analysis with an electrophoretic separation of the restriction fragments in two dimensions and a hybridization with GC-rich minisatellite core probes.

(1) In the first place, Alu, and hence inter-Alu, sequences are distributed across the genome much more regularly than minisatellite-type sequences are (the chance that from a genome fragment of 2 million base pairs not at least 1 inter-Alu fragment can be isolated by means of PCR is very small); the possibly unfavourable melt-out properties of the Alu repeat itself in denaturing gradients does not play a role because we are concerned here for the greater part with inter-Alu regions, with normally only a small part of the Alu sequence itself being taken along.

(2) In the second place, the inter-Alu method enables, after a two-dimensonal separation of the fragments which have been generated with total genomic DNA as substrate, recognition of certain specific regions in the genome by hybridization with a probe mixture consisting of inter-Alu PCR products which come from a specific chromosome (obtained through flow sorting or as hybrid cell line) or a part thereof, for instance in the form of cosmid or YAC clones.

Because it is known that the 3' ends of Alu sequences may have an AT-rich VNTR type polymorphism (Economou et al., 1990; Epstein et al., 1990; Zuliani and Hobbs, 1990) different alleles of an "inter-Alu" locus can be distinguished from each other by length on an isotherm in the denaturing gradient dimension. In a similar way polymorphisms can be detected which are based on the presence or absence of a particular binding site for the Alu-specific primer and/or the presence or absence of a particular restriction enzyme recognition site in the

inter-Alu region (Sinnett et al., 1990). Thus, a 2-D separation pattern of inter-Alu PCR products coming from total genomic DNA of different individuals can directly provide information about genetic differences and similarities, namely specifically for certain chromosomes or parts thereof, depending on the probe mixture used. As probe mixtures may for instance serve inter-Alu products originating from human-hamster hybrid cell lines which contain one or more human chromosomes or parts thereof. The DNAs in question can be labeled and subsequently used as hybridization probe for the analysis of 2-D separation patterns of inter-Alu amplification products (for lack of homology, the hamster DNA will not bind). This will result in an individual-specific stain pattern, each stain being in principle a variant of an inter-Alu DNA fragment which is located in the region that is specified by the human chromosome (or a part thereof) present in the hybrid cell line.

It is also possible to use as probe mixtures inter-Alu products of YAC and cosmid clones coming from reference libraries, the advantage being that a direct link can be made between the physical map (= the location in the sequence order) and the genetic map of the genome (= the location on one of the chromosomes as determined by linkage analysis).

The label used for the probe is not in itself critical and all known labels for DNA or RNA probes can be used. Thus, it is for instance possible for the probe that is used in the hybridization analysis to be labelled with a radioisotope such as ^{32}P and ^{35}S, with a hapten such as biotin and digoxigenin, with a fluorescent substance or with a combination of such labels.

The method described hereinabove can be used in the determination of genetic variation in any animal or plant species, provided that individual chromosomes or parts thereof are available, for instance in the form of flow-sorted chromosomes, hybrid cell lines (in which case the DNA of the other species should have no homology with the "Alu-like" priming sites in the DNA of the species to be examined) and cosmid or YAC clones.

Nor is the method confined to the Alu or Alu-like repeat family, but it can also been used on other human repeat families, such as the Kpn family (see Ledbetter et al., 1990a; see further Table 2 for an overview of Kpn specific primers and Fig. 4 for a diagrammatic overview of the structure of Kpn repeats and the binding sites of the Kpn primers) or repeat families whose location is limited to for instance the centromeres or the telomeres (even of specific chromosomes; see Table 3 for an overview of primers which are specific for a centromere repeat on chromosome 21; see Devilee et al., 1988) and on repeat families in other animal and plant species. Furthermore, it is naturally possible to use combinations of repeats so that inter-repeat regions can be detected which are enclosed between for instance an Alu at one end and a Kpn at the other.

TABLE 2: Origin and sequence of Kpn/LINE specific primers

Unpublished:	
KpnA	5'-CGCGGGCGGCCGC-CCTAATGCTAGATGACGAGTTAGTGGG-3'
KpnB	5'-CGCGGGCGGCCGC-GGGTGCAGCACACCAACATGGC-3'
KpnB1	5'-CGCGGGCGGCCGC-GGGTGCAGCACACAAC-3'
KpnC	5'-CGCGGGCGGCCGC-GGCACATGTATACATATGTAACAAACCTGC-3'
KpnC1	5'-CGCGGGCGGCCGC-CATGGCACATGTATAC-3'
KpnD	5'-CGCGGGCGGCCGC-GCACGTTGTGCACATGTACCCTAGAACTTAA-3'
Ledbetter et al., 1990a:	
L1Hs	5'-CATGGCACATGTATACATATGTAAC(A/T)AACC-3'

TABLE 3: Origin and sequence of human chromosome 21 centromere-specific L1.26 primers

Unpublished:	
L1265a	5'-CGCGGGCGGCCGC-CATTCTCAAGAAACTGCTTTGTGATG-3'
L1265b	5'-CGCGGGCGGCCGC-CATCACAAAGCAGTTTCTTGAGAATG-3'
L1263	5'-CGCGGGCGGCCGC-CATTCTCAAGAAACTGCTTTGTGATA-3'
L126W	5'-CGCGGGCGGCCGC-CATTCTCAAGAACT(T/G)GTT(T/C)(G/C)TGATG-3'
L1263'	5'-CGCGGGCGGCCGC-GGA(G/T)(C/T)GCTTTGACGATTTCG-3'
L126N	5'-CGCGGGCGGCCGC-GACAGAAGCATTCTCAGAAAC-3'

The invention can be used in genetic diagnostics focusing on the detection of DNA marker probes for hereditary characteristics. This is the case in medical diagnostics where the method can be used to screen within a short time a large number of polymorphic sites in a priorly defined region within a chromosome, for individual differences, which information can be used directly to obtain a marker for a hereditary characteristic, such as a hereditary disease. When it is found, for example, that a given variant of an inter-Alu region is invariably inherited together with a specific hereditary characteristic, it is possible to isolate the DNA fragment in question from the hybridization membrane by means of PCR. The fragment in question can then be located in detail. An alternative is to locate beforehand all spots (inter-Alu variants) which can be found in a 2-D chromosome-specific DNA scan and to reduce them to a specific recombinant DNA clone in one of the reference libraries open to the public. The setting up of such a database of polymorphic DNA sequences as spots in a 2-D separation pattern is discussed extensively in Uitterlinden and Vijg, 1989 and EP-A-0349024.

The invention, then, relates to the following:

1. Electrophoretic separation in 2 dimensions (i.e. size and base pair composition) of inter-repeat PCR products obtained from total genomic DNA from a human, animal or plant using one or more primers specific for one or more repetitive sequences.
2. Hybridization of the separation pattern obtained under 1 with a probe mixture consisting of inter-repeat PCR fragments obtained from (1) purified chromosomes, or parts thereof, from the organism that is being studied, and/or (2) genomic DNA originating from somatic cell hybrids having therein as a complement the chromosome or the chromosomes, or parts thereof, of the organism that is being studied, and/or (3) cosmid and/or YAC clones.

The invention can be illustrated in and by the following experiments.

Experiment 1

With a number of genomic DNA samples of different origin as substrate, a PCR reaction was performed, using PCR primer TC65. The primer was synthesized on a Gene Assembler Plus (Pharmacia, Sweden) and was used directly after the deprotection-isolation step, without further purification. The sequence of this primer, consisting of 30 nucleotides, is 5'-AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT-3' (see also Nelson et al., 1989). The PCR reactions were performed in a total volume of 50 µl (microlitre) containing 400 ng genomic DNA, 1 µM primer TC65, 50 mM KCl, 10 mM Tris.HCl pH 8.0, 1.5 mM $MgCl_2$, 200 µM dATP, 200 µM dTTP, 200 µM dCTP, 200 µM dGTP and 1 unit of Taq polymerase (Gibco-BRL, Bethesda, Maryland, USA). Using an automated "thermal cycler" (BIOMED, Germany) a total of 30 cycli were traversed, each cycle comprising successively 2 min denaturation at 95°C, 1 min annealing at 60°C, and 4 min extension at 72°C. The first denaturation step was prolonged by 5 min and the final extension step was prolonged by 4 min. The genomic DNA samples used were Chinese Hamster Ovary (CHO) total genomic DNA and Hela human total genomic DNA, and CHO-human somatic cell hybrid genomic DNA from the following cell lines: 643C-13, 21q+, R2-10W, ACEM, 2Fur-1, 72532X-6, and 153E7b (see Gardiner et al., 1990). Fig. 5A shows the human chromosome 21 complement of these hybrids. After completion of the reaction, 25 µl of the reaction mixture was subjected to electrophoresis on a 1 mm thick 5% polyacrylamide (PAA) gel (acrylamide : bisacrylamide = 37:1), using a gel apparatus as described in Fischer and Lerman, 1979. Electrophoresis (2 h and 250 volts) was done in 1xTAE (40 mM Tris.HCl pH 7.4, 20 mM Na-acetate, 1 mM Na-EDTA) at 50°C. After completion, the separation pattern was visualized by staining with ethidium bromide (0.1 µg per ml) and decolouring in water for 30 min. Fig. 5B shows that the TC65 PCR reaction is specific for human DNA since no colouring occurs in the lane with PCR products of total genomic hamster DNA. In the lane with total genomic human DNA, a smear is visible with a single band therein. This is due to the very large number of different inter-Alu products originating from

all human chromosomes together. In the lanes with the CHO-human cell hybrids, however, discrete bands are visible. Since these hybrids only have the human chromosome 21, these bands must originate from inter-Alu regions on the human chromosome 21. The band patterns are mutually different, which is a reflection of the different pieces of chromosome 21 which the cell line in question contains (see Fig. 5A). In this way specific bands can be identified as originating from specific regions on the chromosome.

Experiment 2

As described under experiment 1, a TC65 PCR reaction was performed, now with genomic DNA of the cell line 72532X6 as a substrate being compared with Hela human genomic DNA. After the PCR reaction, 25 µl of the two reaction products was separated on a PAA gel as described above. After staining of this 1-D separation pattern, the fragments with a length of between 10 kilo base pairs (kb) and 600 base pairs (bp) were excised as lanes and transferred to a 2-D PAA gel. The 2-D gel was a 1 mm thick 6% PAA gel (see above) which contained a 10-75% linear gradient of the denaturants urea and formamide (100% denaturants corresponds to 7.0 M urea, 40% formamide) parallel to the direction of the electrophoresis. The gels were prepared by mixing the two solutions with the two most extreme denaturant concentrations, which determine the range of the gradient, in a gradient mixer (Gibco-BRL) using a peristaltic pump (LKB, Sweden). The electrophoresis for the second dimension separation was performed at 55°C in 1xTAE, for 12 h. After completion of the separation, the 2-D gel was stained as described above (see Fig. 6). The separation pattern of 72532X6 demonstrates that the TC65 inter-Alu PCR fragments focus as spots in the 2-D gel. At the place where the TC65 inter-Alu PCR products of total genomic human Hela DNA are located, nothing is to be seen on gel because the concentration of individual PCR fragments is too low to be demonstrated by ethidium bromide staining.

To visualize these fragments, the 2-D separation pattern was transferred to a nylon membrane (Hybond N-plus, Amersham, UK) by means of electroblotting between horizontal graphite plates with the anode at the top (see also Vijg and Uitterlinden, EP-A-0 349 024). For this purpose, the 2-D gel was first irradiated for 4 min with UV light of 302 nm (Transilluminator, UV products, CA, USA). The gel was placed on the nylon membrane and subsequently placed on opposite sides between three Whatmann 3MM paper leaves which had been saturated with 1xTBE (89 mM Tris.borate pH 8.0, 89 mM boric acid, 2 mM Na-EDTA). This sandwich was placed between the graphite plates and the electroblot procedure was performed for 1 h with the voltage running up from 6 to 28 V and the current being kept constant at 400 mA. After the electroblotting, the nylon membrane was placed on a leaf of Whatmann 3MM which had been saturated with 0.4 N sodium hydroxide and subsequently placed in the 1 M Tris.HCl pH 8.0 for 10 min for neutralization, so as to make the DNA fragments on the membrane single-stranded. The membrane was dried in air and subsequently irradiated with 302 nm UV to bind the DNA fragments to the membrane by cross-linking. This membrane was subsequently subjected to hybridization analysis wherein as a probe use was made of 3 µl of the TC65 inter-Alu PCR mixture obtained with 72532X6 as substrate (and of which 25 µl in the first dimension was separated on gel, see Figs. 5 and 6). These fragments were labelled with α^{32}P-dCTP in the random prime labelling (Feinberg and Vogelstein, 1984). For use in the hybridization, this labelled probe mixture was made single-stranded by boiling it for 5 min and it was subsequently preincubated with 200 µg partly degraded human DNA in a total volume of 1 ml at 65°C for 1 h, so as to repress cross hybridization with the Alu repeats themselves and to allow only the inter-Alu sequences to hybridize.

Hybridization analysis was performed in glass tubes in a hybridization oven (GFL, Germany) at 65°C. The membrane was first preincubated for 15 min in the hybridization fluid which consisted of 7% SDS, 0.5 M Na-phosphate pH 7.2, 1 mM Na-EDTA.

Then the labelled and preincubated 72532X6 TC65 inter-Alu probe was added and the hybridization reaction took place for 16 h. Then the membrane was successively washed in 2.5 x SSC/0.1% SDS, 1 x SSC/0.1% SDS, and 0.1 x SSC/0.1% SDS (1 x SSC corresponds to 150 mM NaCl and 15 mM Na-citrate) at 65°C for 30 min for each washing step. Then a Kodak XAR film was exposed to the membrane for 5 h and then for 48 h at -80°C. Fig. 7 shows that after hybridization the same separation pattern for the 72532X6 TC65 inter-Alu PCR products was visualized as what we observed after ethidium bromide staining. Of greater importance is the fact that we can also observe spots at the place of the Hela TC65 inter-Alu products. This demonstrates that it is possible to visualize certain inter-Alu products in an inter-Alu PCR reaction on total genomic human Hela DNA by means of hybridization with labelled inter-Alu products. The location of the spots in the Hela pattern corresponds to that in the 72532X6 pattern.

In a comparable experiment (see Fig. 8) we analyzed human total genomic inter-repeat PCR products obtained with primer TC65. These products were separated by 2-D electrophoresis and the separation pattern was transferred to a nylon membrane and hybridized with radioactively labelled inter-repeat PCR products of total genomic DNA. The inter-repeat PCR products used for separation in the 2-D gel and for hybridization analysis were identical. Fig. 8 shows that multiple spots were visible after autoradiography in the 2-D separation pattern of inter-repeat products obtained from human total genomic DNA, indicating the possibility to scan several different inter-repeat sequences in the human genome.

Experiment 3

The analyses described under experiments 1 and 2 were performed with a primer (TC65) which is specific for the 3' end of the Alu repeat and which yields an inter-Alu product when the flanking Alu repeats are disposed in a particular inverted orientation. However, it is also possible to chose a primer (517) which is specific for Alu and which yields inter-repeat

PCR products when the flanking Alu repeats have exactly the reversed orientation (see also Figs. 2 and 3). Further, comparable PCR products can be obtained when primers are selected for repeats such as the Kpn I repeats (see Table 3). They have a similar distribution through the genome and a comparable copy number. Using the Kpn primers described in combination with the Alu primers, PCR experiments were performed under comparable conditions and likewise band patterns were obtained which are comparable with those obtained with the Alu primers alone (see Figs. 9 and 10).

Experiment 4

Besides primers which are specific for repeats which occur scattered through the genome, inter-repeat primers can be used which are specific for repeats located in the centromeres, such as the alphoid satellite repeats (L1.26 and others). When these primers are used in combination with Alu and/or Kpn primers in experiments similar to experiments 1, 2 and 3, band patterns can be obtained (see Fig. 10) which are comparable with those shown in Figs. 5 and 9.

Captions with the figures

Fig. 1

Two-dimensional separation pattern, generated using minisatellite core probe 33.6 of human HaeIII DNA restriction fragments originating from a mother (circle), father (square on the right) and their son (square in the middle).

Fig. 2

Schematic overview of the principle of the inter-Alu PCR amplification using primers TC65 and 517 (see Nelson et al., 1989).

Fig. 3

Schematic overview of the binding sites of the Alu-specific primers on the Alu repeat.

Fig. 4

A. Schematic overview of the structure and copy number in the human genome of Kpn/LINE repetitive sequences.

B. Schematic overview of the binding sites of the Kpn/LINE-specific PCR primers.

Fig. 5

A. Schematic overview of the composition as to the chromosome 21 contents of human-hamster somatic cell hybrids used for the inter-repeat PCR amplifications.

B. Ethidium bromide-stained one-dimensional separation pattern of the PCR products, obtained after an inter-Alu PCR amplification using primer TC65 on the members of the somatic hybrid panel shown in 5A and on total hamster genomic DNA and totale human genomic DNA.

Fig. 6

Ethidium bromide-stained two-dimensional separation pattern of inter-Alu PCR amplification products, obtained with primer TC65 from the somatic cell hybrid 72532X6.

Fig. 7

Autoradiograms (A is the short and B is the long irradiation) of a two-dimensional separation pattern of inter-Alu PCR amplifications with primer TC65 of human total genomic DNA (Hela) and of the somatic cell hybrid 72532X6. Arrows designate spots in the Hela separation pattern after the long irradiation, which can be recognized at a similar location in the 72532X6 pattern.

Fig. 8

Autoradiograph of a two-dimensional separation pattern of inter-Alu PCR amplification products obtained with primer TC65 of human total genomic DNA (CEPH 0202) and of the somatic cell hybrid 153E7b.

Fig. 9

Ethidium bromide-stained one-dimensional separation pattern of inter-repeat PCR amplification products, obtained with a combination of the Alu-specific primer TC65 with the Kpn-specific PCR primers KpnA, -B, -C, and -D, the substrate

being total hamster genomic DNA and DNA from the somatic cell hybrid 72532X6.

Fig. 10

Ethidium bromide-stained one-dimensional separation pattern of inter-repeat PCR amplification products, obtained with the Alu-specific primers 517 and TC65 in combination with the Kpn-specific primers KpnA, -B, -C, and -D and in combination with the chromosome 21-specific centromere primers 26.5B, 26W and 26.3, the substrate being total hamster genomic DNA and DNA from the somatic cell hybrid 153E7b.

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CLAIMS

1. A method of detecting genetic variation by subjecting DNA fragments, derived from a DNA sample to be analyzed, to a two-dimensional electrophoretic separation, with the fragments being separated in one dimension on the basis of differences in length and in the other dimension on the basis of differences in base sequence, transferring the resultant separation pattern to a membrane filter and subjecting the transferred separation pattern to a hybridization analysis using one or more DNA or RNA probes, characterized by using DNA fragments which consist of inter-repeat sequences which on the basis of the DNA to be analyzed, have been generated by means of a DNA amplification process such as a polymerase chain reaction (PCR) with repeat-specific primer(s).

2. A method according to claim 1, characterized by using as a probe in the hybridization analysis, one or more inter-repeat sequences which have been generated by means of a DNA amplification process such as a PCR with repeat-specific primer(s), performed on subgenomic DNA, for example DNA of one chromosome or a part thereof.

3. A method according to claim 2, characterized in that the subgenomic DNA has been obtained from total genomic DNA by flow-sorting.

4. A method according to claim 2, characterized in that the subgenomic DNA originates from a hybrid cell line.

5. A method according to claim 2, characterized in that the subgenomic DNA originates from a cosmid or yeast artificial chromosome (YAC) clone.

6. A method according to one or more of the preceding claims, characterized by using inter-Alu sequences which have been generated by means of a DNA amplification process such as a PCR with Alu-specific primer(s).

7. A method according to claim 6, characterized by using in the DNA amplification process such as a PCR, Alu-specific primer(s), selected from the group consisting of TC65, 517,

PDJ33, PDJ34, PDJ33A, PDJ34A and PRIM IV.

8. A method according to one or more of claims 1-5, characterized by using inter-Kpn sequences which have been generated by means of a DNA amplification process such as a PCR with Kpn-specific primer(s).

9. A method according to claim 6, characterized by using in the DNA amplification process such as a PCR, Kpn-specific primer(s), selected from the group consisting of KpnA, KpnB, KpnB1, KpnC, KpnC1, KpnD and L1Hs.

10. A method according to one or more of claims 1-5, characterized by using inter-repeat sequences located between an Alu-repeat and a Kpn-repeat, which have been generated by means of a DNA amplification process such as a PCR with an Alu-specific and a Kpn-specific primer.

11. A method according to claim 10, characterized by using in the DNA amplification process such as a PCR, an Alu-specific primer, selected from the group consisting of TC65, 517, PDJ33, PDJ34, PDJ33A, PDJ34A and PRIM IV, and a Kpn-specific primer, selected from the group consisting of KpnA, KpnB, KpnB1, KpnC, KpnC1, KpnD and L1Hs.

12. A method according to one or more of claims 1-5, characterized by using inter-centromere repeat sequences which have been generated by means of a DNA amplification process such as a PCR with centromere repeat-specific primer(s).

13. A method according to claim 12, characterized by using in the DNA amplification process such as a PCR, a centromere repeat-specific primer, selected from the group consisting of L1265a, L1265b, L1263, L126W, L1263' and L126N.

14. A method according to one or more of claims 1-5, characterized by using inter-telomere repeat sequences which have been generated by means of a DNA amplification process such as a PCR with telomere repeat-specific primer(s).

15. A method according to one or more of the preceding claims, characterized in that the probe which is used in the hybridization analysis is labelled with a radioisotope such as ^{32}P and ^{35}S, with a hapten such as biotin and digoxigenine, and/or with a fluorescent substance.

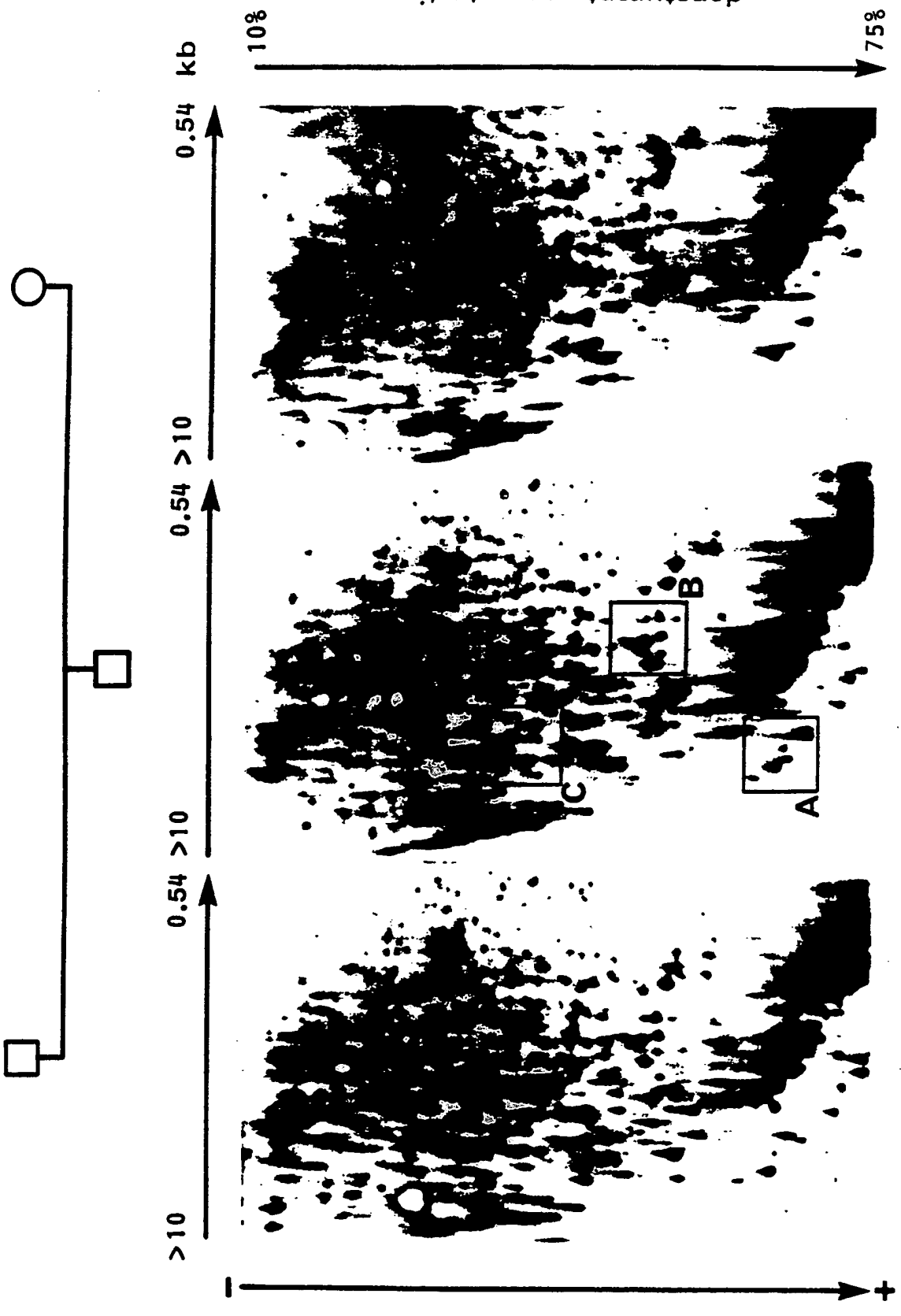
denaturant concentration
10%
75%
0.54 kb
0.54 >10
0.54 >10
>10
electrophoresis
-
+
A
B
C
probe 33.6

FIG. 1

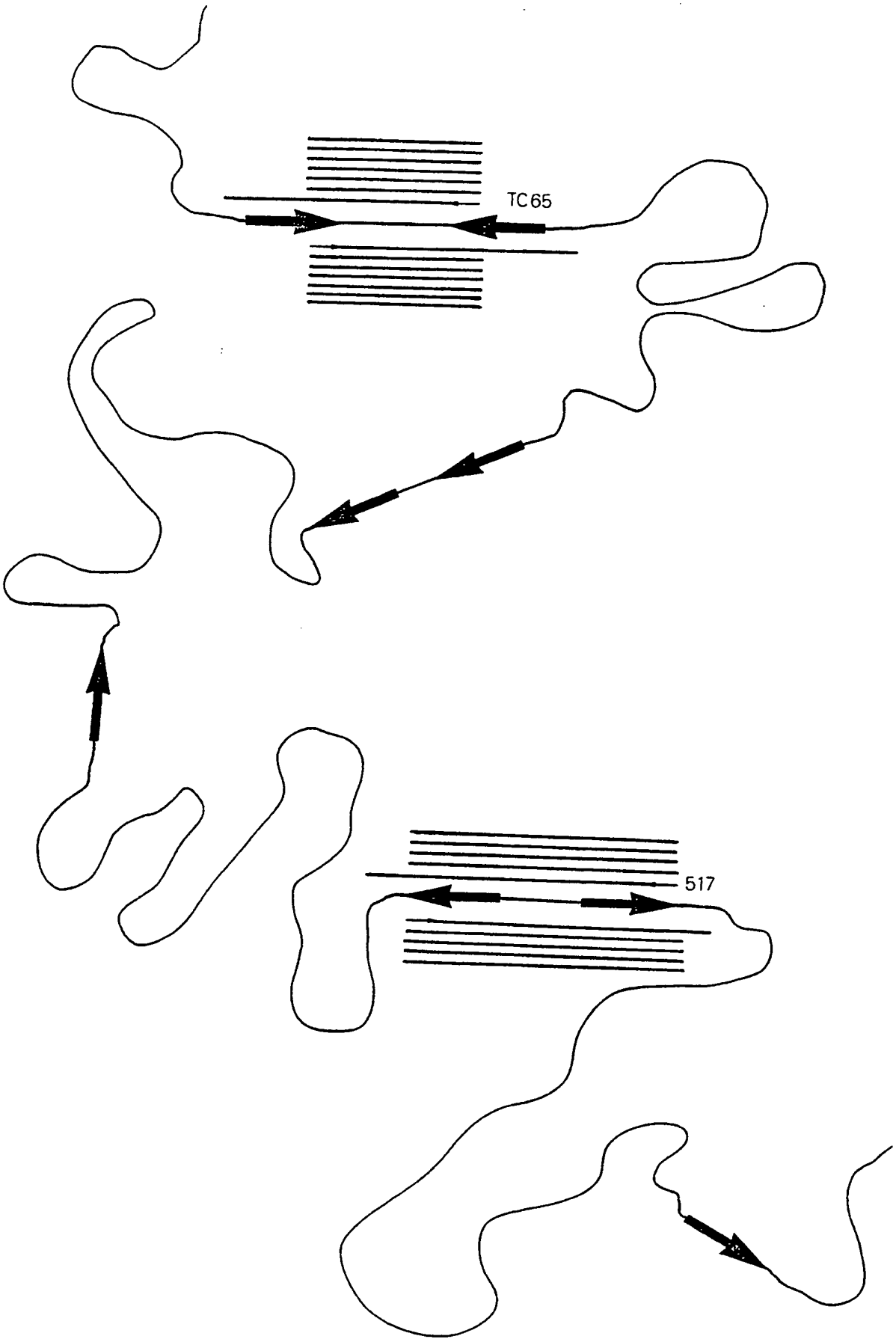

FIG. 2

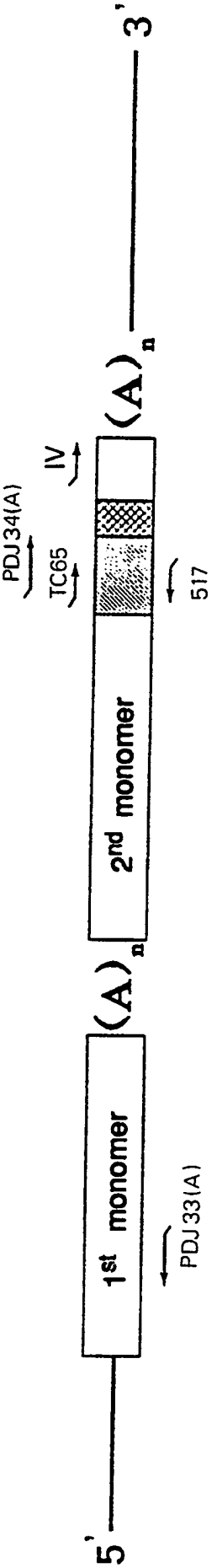

FIG. 3

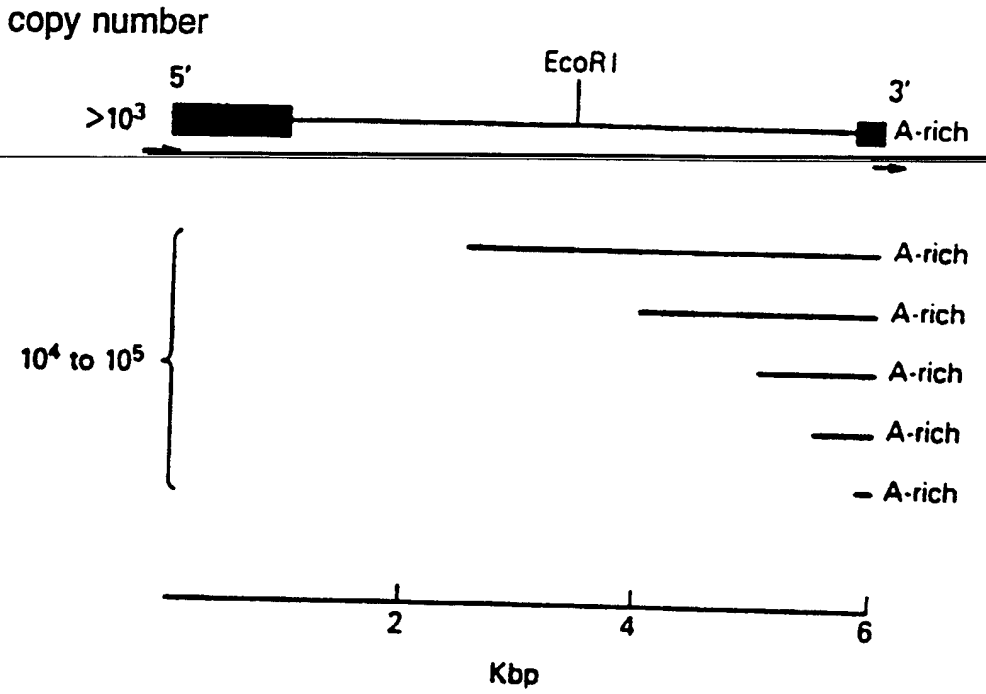
copy number
5'
EcoR I
3'
>10^3
A-rich
10^4 to 10^5
A-rich
A-rich
A-rich
A-rich
A-rich
2
4
6
Kbp

FIG.4A

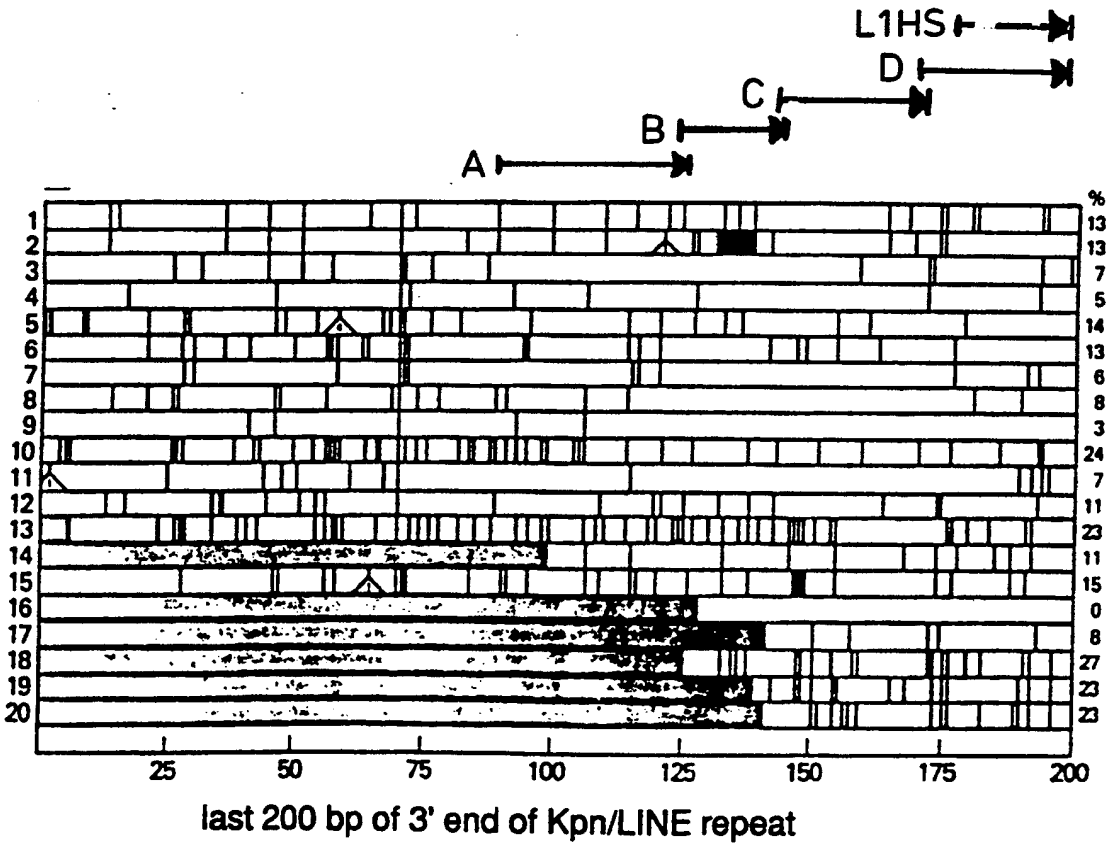
L1HS
D
C
B
A
%
1 13
2 13
3 7
4 5
5 14
6 13
7 6
8 8
9 3
10 24
11 7
12 11
13 23
14 11
15 15
16 0
17 8
18 27
19 23
20 23
25
50
75
100
125
150
175
200
last 200 bp of 3' end of Kpn/LINE repeat

FIG. 4B

FIG.5A

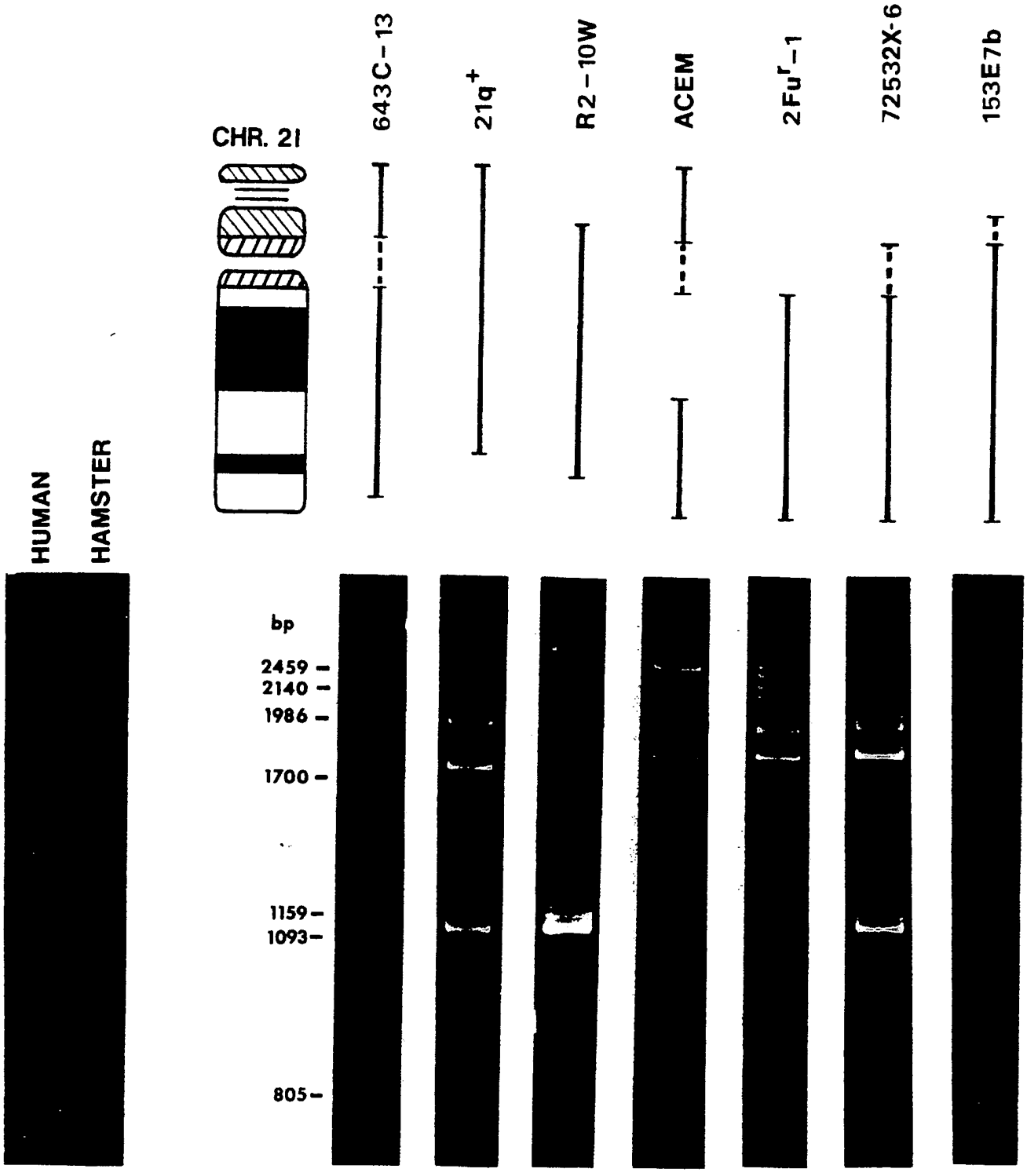
CHR. 21
643C-13
21q+
R2-10W
ACEM
2Fur-1
72532X-6
153E7b
HUMAN
HAMSTER
bp
2459
2140
1986
1700
1159
1093
805
TC 65

FIG.5B

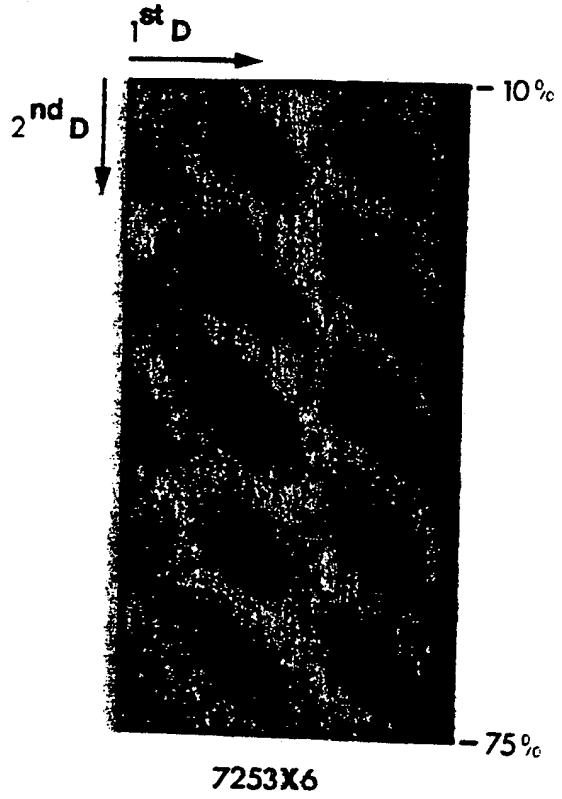
1st D
2nd D
10%
75%
7253X6

FIG.6

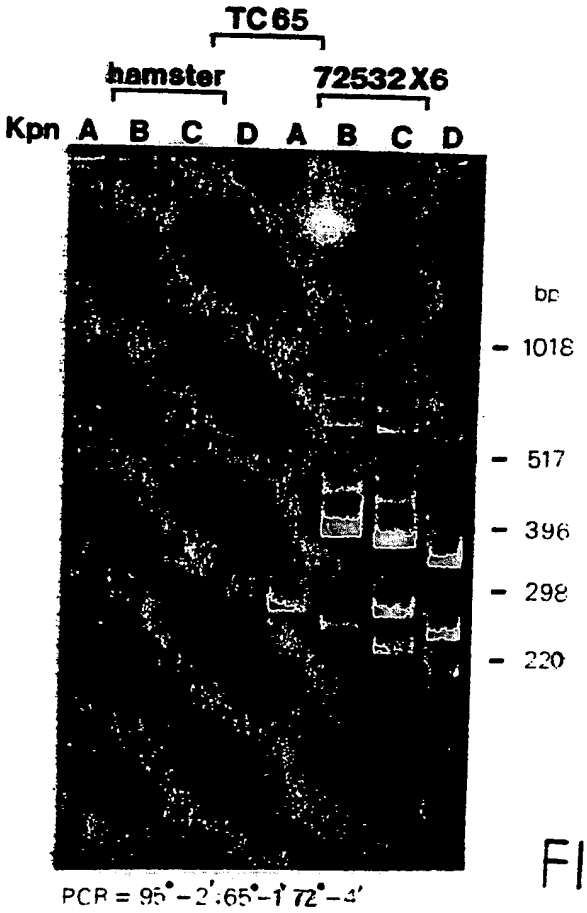
TC65
hamster
72532X6
Kpn A B C D A B C D
bp
1018
517
396
298
220
PCR = 95°-2':65°-1' 72°-4'

FIG.9

HELA

72532X6

FIG.7A

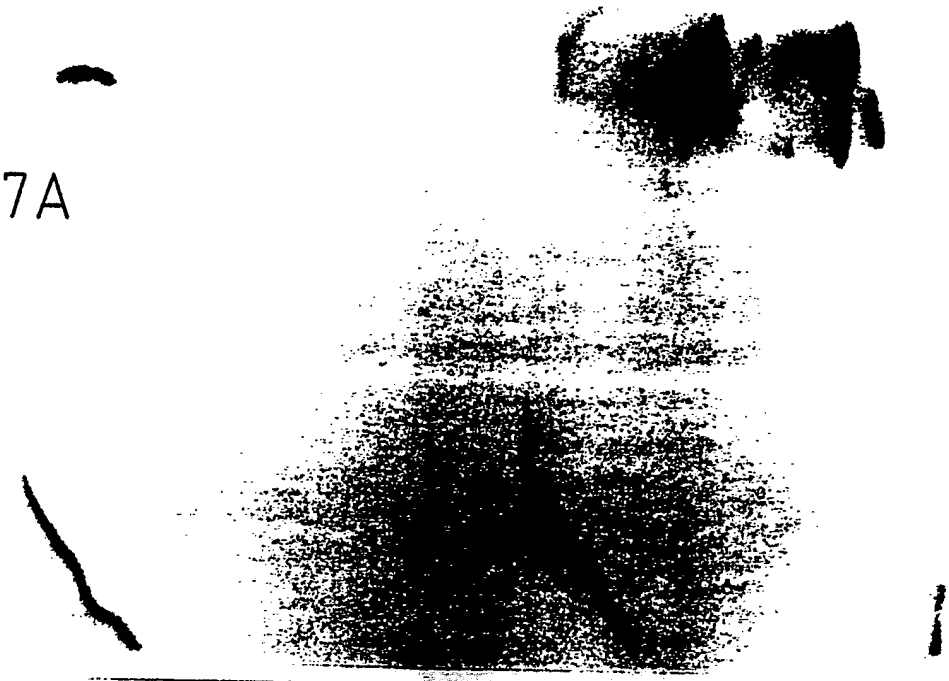

FIG.7B

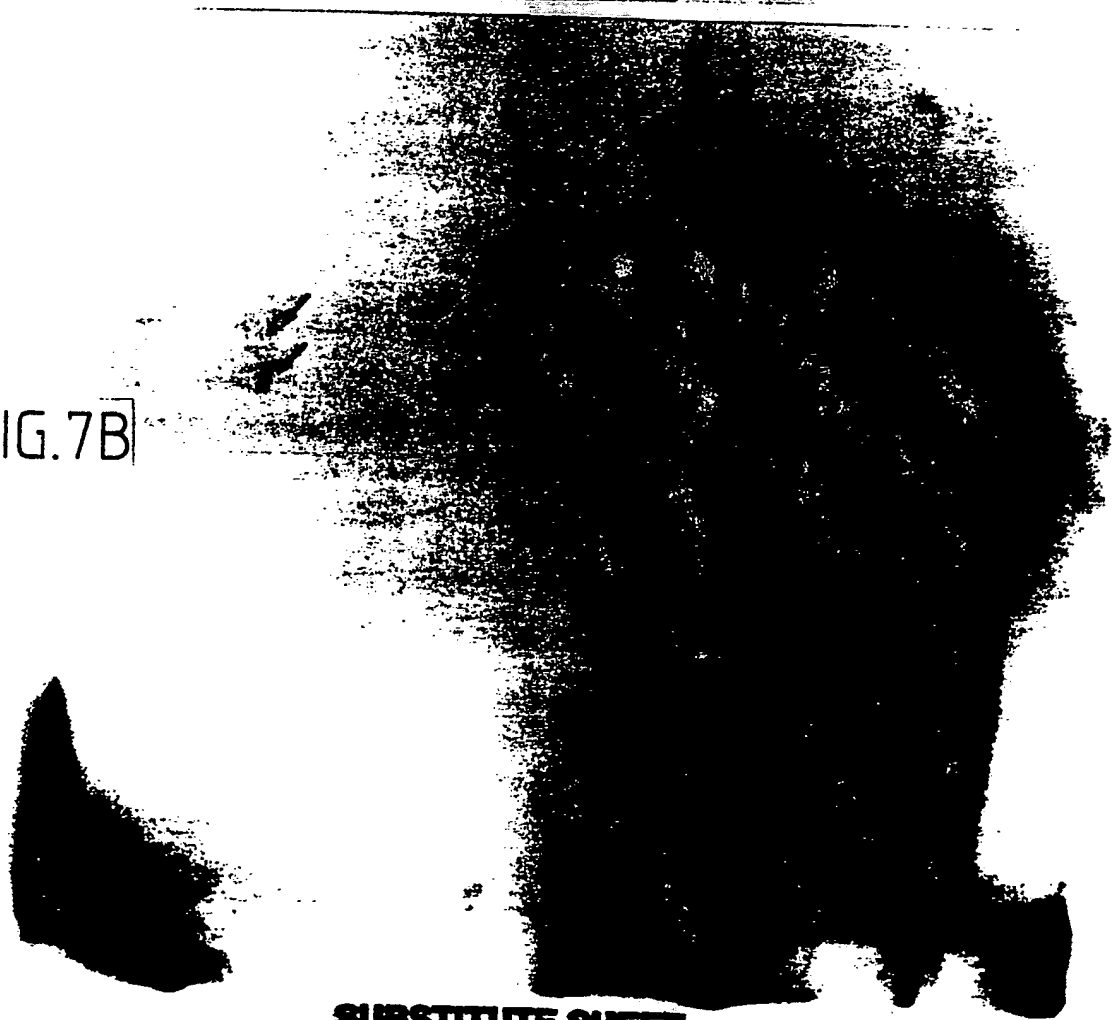

FIG.8

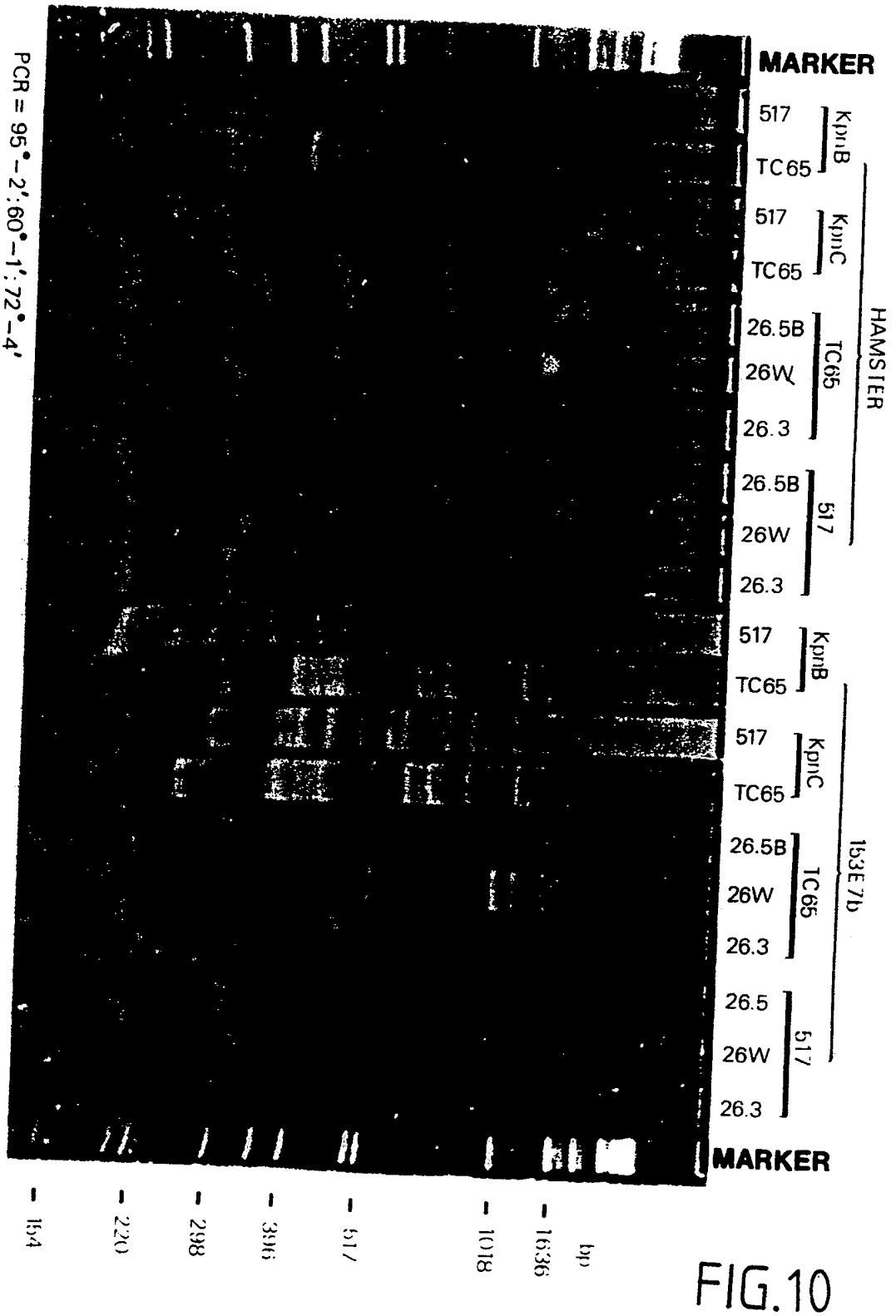
PCR = 95°-2';60°-1';72°-4'
MARKER
517
TC65
KpnB
517
TC65
KpnC
26.5B
26W
26.3
TC65
HAMSTER
26.5B
26W
26.3
517
517
TC65
KpnB
517
TC65
KpnC
26.5B
26W
26.3
TC65
153E7b
26.5
26W
26.3
517
MARKER
154
220
298
396
517
1018
1636
bp

FIG.10

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Nucleic Acids Research, vol. 18. no. 10, 16 March 1990, C. BREUKEL et al.: "Vector-Alu PCR: a rapid step in mapping cosmids and YACs", page 3097, see the whole document	1-6
Y	EP,A,0349024 (NEDERLANDSE ORGANISATIE VOOR TNO) 3 January 1990, see the whole document (cited in the application)	1-6
Y	Proc. Natl. Acad. Sci. USA, vol. 86, April 1989, A.G. UITTERLINDEN et al.: "Two-dimensional DNA fingerprinting of human individuals", pages 2742-2746, see the whole document (cited in the application)	1,2,5-7 ,15

-/-

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16-04-1992	23. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	Proc. Natl. Acad. Sci. USA, vol. 86, September 1989, D.L. NELSON et al.: "Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources", pages 6686-6690, see page 6686 (cited in the application)	1,2,5-7 ,15
Y	Proc. Natl. Acad. Sci. USA, vol. 87, September 1990, P. LICHTER et al.: "Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines", pages 6634-6638, see the whole document (cited in the application)	1,2,5,6 ,8-11
Y	Tibtech, vol. 7, December 1989, Elsevier Science Publishers Ltd, (GB), A.G. UITTERLINDEN et al.: "Two-dimensional DNA typing", page 336-341, see page 339 (cited in the application)	1,2,5,6 ,8-11
Y	Genomics, vol. 6, no. 3, March 1990, Academic Press, Inc., S.A. LEDBETTER et al.: "Rapid isolation of DNA probes within specific chromosome regions by interspersed repetitive sequence polymerase chain reaction", pages 475-481, see page 476 (cited in the application)	1,2,5,6 ,8-11

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. NL 9200018 SA 56350

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/05/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0349024	03-01-90	NL-A- 8801147	01-12-89
		JP-A- 2104299	17-04-90
		US-A- 5068176	26-11-91